Kidney International, Vol. 36 (1989), pp. 519–525

EDITORIAL REVIEW

Regulation of the renal Na^+-H^+ exchanger by protein phosphorylation

Cation exchange transport systems are implicated in the regulation of a number of cell functions. Recent studies indicate that precise regulation of these transporters is critical to cell homeostasis. Malregulation of these transport systems has been linked to the pathogenesis of certain disease states. The present review focuses on the Na^+-H^+ exchanger, an electroneutral transporter which mediates the transmembrane countermovement of sodium and hydrogen ions. Although operationally defined as a sodium for hydrogen exchanger, this transporter also has specificity for other cations. This exchanger is inhibited by amiloride and its analogues. Thus, in many experiments, the activity of the Na^+-H^+ exchanger is defined as the amiloride inhibitable component of proton gradient-stimulated sodium uptake. Another general characteristic of the Na^+-H^+ exchanger common to many cell types is the presence of a pH-sensitive internal modifier site. Several comprehensive reviews of the properties of this transporter have recently been published [1–3].

The Na^+-H^+ exchanger was initially identified in brush border membrane vesicles from small intestine and the proximal tubule of the kidney [4]. More recent studies suggest that many, if not all mammalian cells, possess a Na^+-H^+ exchanger. In the kidney, the Na^+-H^+ exchanger is important in the reclamation of filtered bicarbonate. The transporter is present in the brush border membrane of the proximal convoluted tubule where it mediates the coupling of sodium entry from the lumen into the cell to the secretion of protons from the cell into the lumen [5, 6]. Other nephron segments also have this transport system in the apical membrane of the cells [7, 8]. Abnormalities in the activity of the Na^+-H^+ exchanger have been suggested to be related to some forms of hypertension and, in patients with diabetes mellitus, correlated with the development of diabetic glomerulosclerosis [9–11]. In cells not directly involved in regulation of the overall fluid and electrolyte balance of the organism, Na^+-H^+ exchange transport appears to be activated in defense of cell pH and/or cell volume [12, 13]. Intense investigative study has recently focused on activation of Na^+-H^+ exchange activity as a necessary step in stimulated cell growth [13, 14].

The Na^+-H^+ exchanger is a regulated transported system. As reviewed by Grinstein and Rothstein, this transporter is subject to two general types of regulation which are identified as short-term and long-term regulatory influences [3]. The present review focuses specifically on short term regulation of the Na^+-H^+ exchanger of the renal proximal tubule brush border membrane by protein phosphorylation mediated by protein kinases which can act as regulators of cell function.

As background for the ensuing discussion, a brief summary of some methodological considerations related to study of the Na^+-H^+ exchanger is helpful. Bicarbonate reabsorption in the proximal convoluted tubule can be measured by micropuncture techniques and by in vivo microperfusion of isolated proximal convoluted tubules [15, 16]. While these types of experiments have yielded important information, it is now known that bicarbonate reabsorption in the proximal tubule is not solely the consequence of hydrogen ion secretion from cell to lumen mediated by the Na^+-H^+ exchanger [17]. The Na^+-H^+ exchanger is a secondary active transporter which harnesses energy from the transmembrane sodium gradient, such that changes in ion gradients may influence the rates of transport activity. In intact cells, it is often difficult to quantify such gradients and/or control them with certainty. Studies in native renal brush border membrane vesicles have the advantage of permitting the investigator to set the initial conditions of the experiment. The disadvantage of using native membranes for study of short term regulation of the Na^+-H^+ exchanger is that soluble cytosolic components and/or loosely associated membrane proteins which may contribute to the regulatory response may not be present in the vesicle preparation. In addition, the preparation procedures which select for right-side-out oriented vesicles may limit access of added factors to the cytosolic surface of these membranes. Experimental protocols have been developed, however, which can alleviate many of these concerns and have greatly facilitated the investigation of the molecular mechanisms involved in the regulation of the Na^+-H^+ exchanger [18–20].

The complexity of the polypeptides present in intact renal proximal tubular cells and in isolated brush border membranes from these cells hampers the unequivocal interpretation of the molecular processes under examination. The use of detergent solubilized, renal brush border membranes permits the segregation and characterization of the proteins involved and offers the opportunity to define the structure and regulation of the Na^+-H^+ exchanger. We have recently reported a series of experiments whereby the proteins of the rabbit renal brush border membrane were solubilized with detergent and Na^+-H^+ exchange activity assayed after reconstitution of the proteins into artificial lipid vesicles [21]. As compared to liposomes containing no proteins, proteoliposomes reconstituted with solubilized renal brush border membrane proteins demonstrated five- to sevenfold higher rates of proton gradient-stimulated sodium uptake. Figure 1 demonstrates that chemically voltage clamped, reconstituted proteoliposomes have higher rates of sodium uptake under proton gradient as com-

Received for publication September 6, 1988
and in revised form November 28, 1988
Accepted for publication December 20, 1988

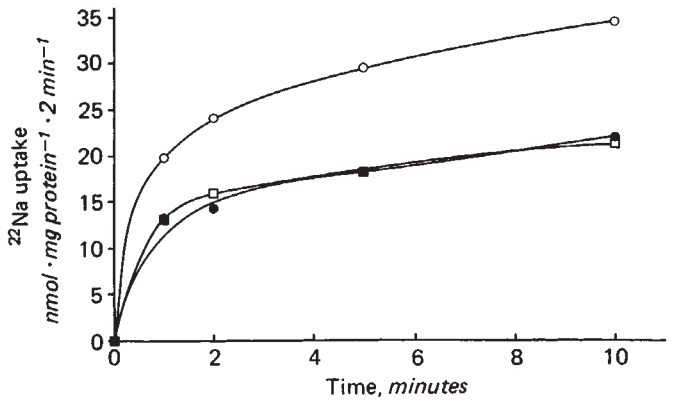

Fig. 1. *The uptake of 1 mM sodium in proteoliposomes reconstituted with rabbit brush border membrane proteins.* Brush border membrane proteins were solubilized with the detergent octyl glucoside and reconstituted into artificial soybean phospholipid vesicles after overnight dialysis. The time dependent uptake of 1 mM sodium was determined in voltage clamped ($K_{in}^+ = K_{out}^+ = 30$ mM, valinomycin 1 μg/ml) proteoliposomes in the presence of an outwardly-directed proton gradient, (○) ($pH_{in} = 6.0$, $pH_{out} = 8.0$), in the absence or presence of 1 mM amiloride (□) and in the absence of a proton gradient, (●) ($pH_{in} = pH_{out}$ 6.0).

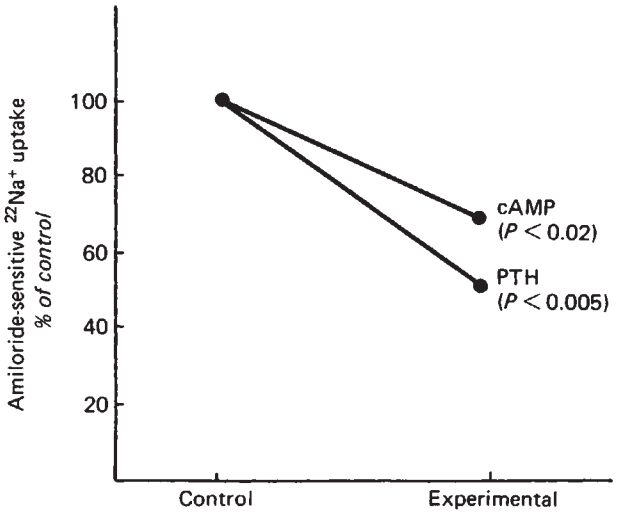

Fig. 2. *The effect of parathyroid hormone (PTH) and cAMP on Na^+-H^+ exchange activity in rabbit proximal convoluted tubules.* An enriched suspension of rabbit proximal convoluted tubules was incubated in absence or presence of either PTH or dibutyryl cAMP for 30 min. Brush border membrane vesicles were prepared from the suspended tubules. The amiloride (1 mM) sensitive component of proton gradient ($pH_{in} = 5.7$, $pH_{out} = 7.4$) stimulated uptake of 1 mM sodium was determined in brush border membrane vesicles. Results are presented as percent change from controls. (Adapted from reference 29).

pared to non-proton gradient conditions and that amiloride inhibits the proton gradient-stimulated component of sodium uptake. Other experiments with proteoliposomes reconstituted with rabbit renal brush border membrane proteins have indicated that proton gradient-stimulated sodium uptake bears many of the properties characteristic of the exchanger in native brush border membrane vesicles. The use of solubilized proteins and the ability to demonstrate activity after reconstitution has provided some new insights on the regulation of the Na^+-H^+ exchanger and on the structure of this transporter.

Regulation of the Na^+-H^+ exchanger by cyclic adenosine monophosphate-dependent protein kinase

In 1971, a series of renal micropuncture studies were performed to examine the effect of parathyroid hormone and cAMP on the renal handling of phosphate [22]. While not a prime objective of these studies, the results indicated that both the hormone and its presumed intracellular mediator inhibited water absorption in the proximal tubule. Studies by others have repeatedly confirmed this observation. Prior in vivo microperfusion studies in the rat proximal tubule from our laboratories, for example, have indicated that inclusion of a permeant cAMP analogue in the luminal perfusion solution decreased the rate of water absorption by 35% [23]. Clearance studies in the dog demonstrated that cAMP decreased the tubular maxima for bicarbonate reabsorption [24]. More direct in vivo microperfusion studies in the rat and in vitro microperfusion studies in the rabbit indicated that parathyroid hormone, acting by the generation of cAMP, inhibited bicarbonate reabsorption in the proximal tubule [15, 25]. While these results are consistent with the interpretation that cAMP inhibited the activity of the Na^+-H^+ exchanger, other explanations have been suggested. Since parathyroid hormone and cAMP have been proposed to increase the leakage of markers through the paracellular pathway, it is possible that the observed decrease in the net reabsorption of bicarbonate was the result of enhanced backleak [26, 27]. Other experiments have raised the possibility that parathyroid hormone inhibits renal carbonic anhydrase activity [28]. Direct evidence that parathyroid hormone and cAMP inhibits the Na^+-H^+ exchanger was derived from studies using an enriched suspension of rabbit proximal convoluted tubules [29]. The tubule suspension was incubated in parathyroid hormone or a permeant analogue of cAMP. Brush border membrane vesicles were prepared from the suspended tubules and Na^+-H^+ exchange activity examined under the same initial conditions. The results of these studies are summarized in Figure 2. As compared to controls, the amiloride sensitive component of proton gradient-stimulated sodium uptake in brush border membrane vesicles obtained from tubules exposed to either parathyroid hormone or cAMP was significantly lower. Sodium-dependent glucose transport was not affected by parathyroid hormone or by cAMP. These results indicate the capacity of parathyroid hormone and/or cAMP to modulate the activity of the renal brush border Na^+-H^+ exchanger. These experimental findings were confirmed independently in studies using cultured proximal tubule cells [30].

The major cellular, high affinity binding protein for cAMP is cAMP-dependent protein kinase (PKA). PKA is a heterodimer consisting of two regulatory and two catalytic subunits and is present in the cytosol of renal proximal tubule cells. Recent studies from the laboratory of Hammerman in the dog and from our laboratories in the rabbit have indicated that PKA is also an intrinsic renal proximal tubule apical membrane protein [31, 32]. In both species, membrane bound PKA appears to be the type II isoenzyme. The presence of PKA in the renal brush border membrane suggests that membrane vesicles themselves may be used to study the relation between PKA-mediated protein phosphorylation and the activity of the Na^+-H^+ ex-

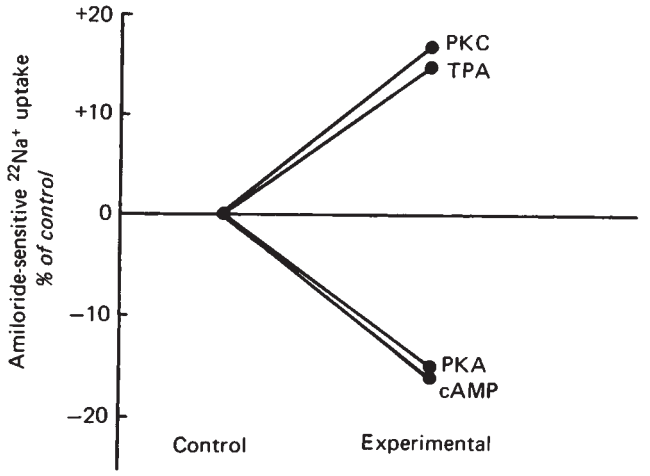

Fig. 3. *Summary of the effects in vitro protein phosphorylation of rabbit brush border membranes by endogenous or exogenous PKA or PKC on Na^+-H^+ exchange activity.* Brush border membrane vesicles were rendered permeable by incubation in a hypotonic phosphorylating solution. The vesicles were resealed and the amiloride (1 mM) sensitive component of protein gradient (pH_{in} = 5.7, pH_{out} = 7.4) uptake of 1 mM sodium determined. For the study of PKA, the phosphorylating solutions contained ATP and magnesium (Control) and either cAMP to stimulated membrane bound PKA or exogenous catalytic subunit of PKA (Experimental). For the study of PKC, calcium was added to the control solution. The experimental solutions contained either the phorbol ester, TPA, to stimulate membrane bound PKC or exogenous PKC. Results are expressed as percent change from control. (Adapted from references 18 and 19).

changer. Rabbit renal brush border membrane vesicles were rendered permeable by incubation in a hypotonic solution containing magnesium, ATP, and either cAMP or purified catalytic subunit of PKA [18]. As summarized in Figure 3, cAMP acting through endogenous membrane bound PKA or exogenous catalytic subunit of PKA inhibited the activity of the Na^+-H^+ exchanger. For example, cAMP inhibited the amiloride sensitive component of sodium uptake from 2.48 ± 0.39 nmol · 10 sec^{-1} · mg $protein^{-1}$ in controls to 2.16 ± 0.33 ($P < 0.01$); a change of approximately 15%. The inhibitory effect of PKA required phosphorylation of some protein component present on the inner cytosolic face of the membrane, had an absolute requirement for ATP, and was inhibited by the specific protein inhibitor of PKA. One concern in the above studies was that, despite the statistically significant decrease in Na^+-H^+ exchange activity following PKA-mediated protein phosphorylation, the magnitude of the observed change was small. Additional experiments indicated that the change in activity of the Na^+-H^+ exchanger was not the consequence of changes in buffering capacity of vesicles or in the rates of passive dissipation of the sodium or hydrogen ion gradients. In addition, the concentrations of ATP, cAMP, and catalytic subunit of PKA were not rate limiting in these experiments. The reason for the relatively small response to protein phosphorylation mediated by cAMP-dependent protein kinase is unknown at present. Based on preliminary data from our laboratories [33], it might be speculated that preparation of brush border membrane vesicles is associated with partial proteolysis of an inhibitory component of the Na^+-H^+ exchanger, a component whose inhibitory activity is stimulated by PKA-mediated protein phosphorylation. Nonetheless, these findings in native brush border membranes provide experimental support for the conclusion that the activity of the Na^+-H^+ exchanger can be regulated by PKA-mediated protein phosphorylation. SDS-PAGE, and autoradiography indicated that a considerable number of membrane proteins were substrates for PKA. These studies also demonstrated that the native brush border membranes contained associated protein phosphatases which resulted in the rapid dephosphorylation of these substrate proteins.

By contrast to native brush border membranes, detergent solubilized, renal brush border membrane proteins possess less endogenous protein kinase activity and little or no protein phosphatase activity [34]. The reduced protein kinase and protein phosphatase activity in solubilized, brush border membrane proteins facilitates the ability to examine the effects of specific protein kinase-mediated regulation of the Na^+-H^+ exchanger and to characterize the associated membrane phosphoproteins. Recent studies from our laboratories have indicated that PKA-mediated protein phosphorylation of solubilized rabbit, renal brush border membrane proteins resulted in a decrease in the amiloride sensitive component of proton gradient-stimulated sodium uptake when assayed after reconstitution of the proteins into lipid vesicles (Fig. 4) [34]. The effect of PKA had an absolute requirement for ATP and was blocked by the specific protein inhibitor of PKA. At maximal inhibitory concentrations of PKA, the amiloride sensitive component of proton gradient-stimulated sodium uptake was decreased from 7.9 ± 0.6 nmol · mg $protein^{-1}$ · 2 min^{-1} in controls to 5.7 ± 0.4 ($P < 0.01$). The magnitude of change observed, 28%, was approximately twofold that observed in native membrane vesicles. These results extend the findings observed in native renal brush border membrane vesicles. The fact that regulation of the Na^+-H^+ exchanger by PKA-mediated protein phosphorylation remains intact after the process of solubilizing the membrane proteins provides an important foundation for subsequent studies designed to isolate and characterize the structure of the Na^+-H^+ exchanger.

In summary, studies in whole kidney, in proximal tubules, in brush border membranes, and in solubilized, renal brush border membrane proteins assayed after reconstitution all suggest that protein phosphorylation mediated by PKA results in a decrease in the activity of the Na^+-H^+ exchanger. The relatively large number of PKA substrate proteins in renal brush border membranes precluded identification of a unique phosphoprotein which could act as the regulator of the Na^+-H^+ exchanger.

Regulation of the Na^+-H^+ exchanger by protein phosphorylation mediated by calcium-phospholipid-dependent protein kinase

As has been reviewed recently, tumor promoting agents such as phorbol esters stimulate growth in many cells and are associated with alkalinization of the cell cytosol [35–37]. This change in cell pH occurs by a mechanism which requires the presence of sodium in the extracellular media and is inhibited by amiloride. Phorbol esters act via the activation of calcium-phospholipid-dependent protein kinase (PKC), suggesting that the Na^+-H^+ exchanger may be regulated by this protein kinase [38]. Hammerman and co-workers have reported that exposure of dog proximal tubule cells to active phorbol esters was

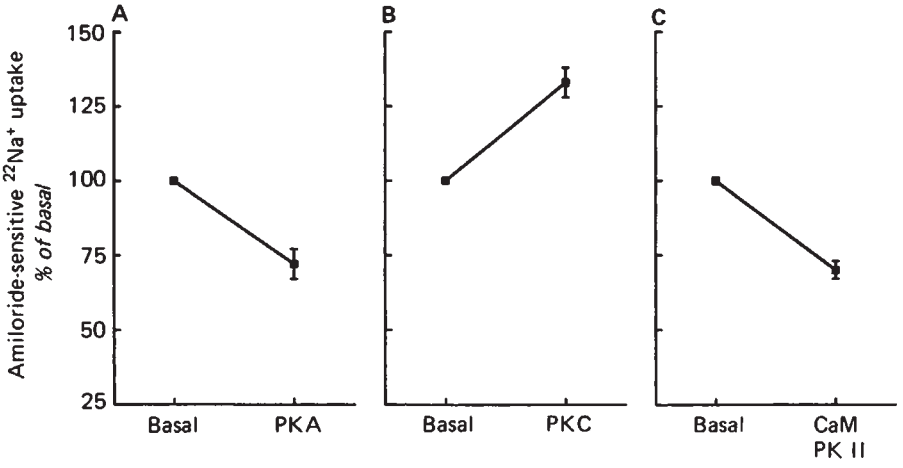

Fig. 4. *Summary of effects of protein phosphorylation of solubilized brush border membrane proteins on Na^+-H^+ exchange activity.* Solubilized brush border membrane proteins were incubated in a phosphorylating solution containing ATP, magnesium, and, where appropriate, calcium, an active phorbol ester, and/or calmodulin (Control) and either catalytic subunit of PKA, PKC, or CaM-kinase II. The proton gradient (pH_{in} = 6.0, pH_{out} = 8.0) stimulated uptake of 1 mM sodium was determined in the absence or presence of amiloride (1 mM) after reconstitution of the proteins into artificial liposomes. Results are expressed as percent change from control. (Adapted from references 34 and 42).

associated with alkalinization of the cell cytosol, and that plasma membranes from the dog proximal tubule contain binding proteins for phorbol esters [39, 40]. PKC is present in the cytosol and brush border membranes of proximal convoluted tubules [41]. To demonstrate a direct effect of PKC on the Na^+-H^+ exchanger, a series of studies was performed in brush border membranes from the rabbit kidney. Using an experimental approach analogous to that described for the study of PKA, it was demonstrated that activation of endogenous PKC by active phorbol esters or the addition of exogenous PKC purified from kidney or brain stimulated the amiloride sensitive component of proton gradient stimulated sodium uptake (Fig. 3) [19]. The stimulatory effect of PKC had an absolute requirement for ATP. Moreover, it was necessary to render the vesicles permeable to obtain a stimulatory response suggesting that PKC mediated the phosphorylation of a protein(s) on the inner, cytosolic face of the membrane. As was the case in the studies of the effects of PKA-mediated protein phosphorylation, the effects of PKC were relatively modest. For example, in one set of experiments, the amiloride sensitive component of proton gradient-stimulated sodium uptake was increased by 15% by PKC-mediated protein phosphorylation from 2.29 ± 0.13 nmol · 10 sec^{-1} · mg $protein^{-1}$ in controls to 2.63 ± 0.15 ($P < 0.01$). The increase in sodium uptake was not the secondary consequence of alterations in the passive permeability of the brush border membranes to sodium or hydrogen ions. The substrate phosphoprotein profile for PKC differed from that of PKA although some common substrates for both kinases were identified.

Additional studies were performed whereby solubilized rabbit, renal brush border membrane proteins were incubated in ATP, calcium, magnesium, partially purified PKC, and an active phorbol ester. Na^+-H^+ exchange transport activity was assayed after the proteins were reconstituted into artificial lipid vesicles [42]. As shown in Figure 4, PKC mediated protein phosphorylation resulted in an increase in the activity of the Na^+-H^+ exchanger. The stimulation of transport activity by PKC had an absolute requirement for ATP, an active phorbol ester, and calcium. At maximally effective concentrations of PKC, ATP, and the phorbol ester TPA, the amiloride sensitive component of proton gradient-stimulated sodium uptake was increased from 6.9 ± 0.5 nmol · 2 min^{-1} · mg $protein^{-1}$ in controls to 9.2 ± 1.1 ($P < 0.01$), an increase of approximately 30%. SDS-PAGE and autoradiography indicated a limited number of substrate proteins for PKC. Interestingly, the number of substrate proteins for PKC in solubilized proteins was fewer than that observed in native membrane vesicles. Collectively, these data provide evidence that protein phosphorylation mediated by PKC stimulated the Na^+-H^+ exchanger in the apical membrane of the rabbit proximal tubule.

Based on the above evidence, it would be predicted that activation of PKC in the proximal convoluted tubule would be associated with an increase in water and bicarbonate reabsorption. Some preliminary data indicates that stimulation of PKC activity in the proximal tubule is associated with enhanced proximal tubule reabsorption of water [43]. In the most detailed report published to date, however, phorbol esters were observed not to increase water or bicarbonate reabsorption in the rabbit proximal tubule [44]. In fact, phorbol esters inhibited the rates of reabsorption. The reasons for this seeming disparity are not known but a number of possibilities might be advanced. It has been suggested that there is a complex interplay between protein kinases which act as regulators of specific processes in intact cells. For example, in some tissues PKC activation has been reported to result in enhanced rates of agonist-stimulated cAMP production [45]. Thus, it is possible that the inhibitory effect of PKA-mediated protein phosphorylation could negate the stimulatory effect of PKC activation. PKC activity is known to rapidly down regulate in cells treated with phorbol esters [46]. An alternate explanation for the lack of stimulation of sodium and bicarbonate reabsorption in renal proximal tubules exposed to phorbol esters is that PKC activity was down regulated during the course of the experiments. Since many of the protein kinases have multiple substrate proteins in the renal proximal tubule cells, the relationship between the activation of a specific protein kinase and a specific cell function examined in intact cells may be extremely complex.

Regulation of the Na^+-H^+ exchanger by protein phosphorylation mediated by calcium-calmodulin-dependent multifunctional protein kinase II

Calcium-calmodulin-dependent multifunctional protein kinase II (CaM-kinase II) appears to be a widely distributed

protein kinase whose physiologic role has yet to be fully delineated. As recently reviewed, this kinase has been isolated and characterized from several tissues [47]. CaM-kinase II activity has been reported in the mammalian kidney although the potential importance of this kinase to renal tubular function has not been examined [48]. By contrast to the study of PKA and PKC where specific activators and/or inhibitors are available to the investigator, study of CaM-kinase II in intact cells is considerably more difficult. Induction of calcium transients may not be an adequately specific signal for this protein kinase. Calmodulin antagonists also lack the necessary specificity required for use in intact cells. To evaluate the potential actions of CaM-kinase II in the kidney, we incubated solubilized rabbit, renal brush border membrane proteins in calcium, calmodulin, magnesium, ATP, and highly purified Cam-kinase II from brain [42]. The activity of the Na^+-H^+ exchanger was assayed after reconstitution. CaM-kinase II-mediated protein phosphorylation resulted in a decrease in the amiloride sensitive component of proton gradient-stimulated sodium uptake (Fig. 4). At maximally effective concentrations of CaM-kinase II, ATP, and calmodulin, the amiloride sensitive component of proton gradient stimulated sodium uptake was decreased from 7.2 ± 0.2 nmol $\cdot$ 2 $min^{-1} \cdot$ mg $protein^{-1}$ in controls to 5.4 ± 0.2 ($P < 0.05$). Maximal inhibition of the Na^+-H^+ exchanger by protein phosphorylation mediated by CaM-kinase II approximated that observed with PKA. The inhibitory effect of CaM-kinase II had an absolute requirement for ATP, calmodulin, and calcium. SDS-PAGE and autoradiography indicated that CaM-kinase II had a unique profile of substrate proteins as compared to either PKA- or PKC-mediated protein phosphorylation. Due to the lack of direct data, it is not possible yet to correlate the physiologic implications of these findings in intact proximal tubule cells.

Physiologic implications of protein phosphorylation mediated by PKA, PKC, and/or CaM-kinase II as it relates the effect of parathyroid hormone on the Na^+-H^+ exchanger

Studies in native renal brush border membranes and in solubilized proteins indicate that protein phosphorylation mediated by PKA and CaM-kinase II inhibits the activity of the Na^+-H^+ exchanger, while that of PKC stimulates the activity of this transporter. Renal proximal convoluted tubules contain receptors for parathyroid hormone in the basolateral membrane [49]. In response to exposure to parathyroid hormone, renal proximal tubule cells produce cAMP [50]. Thus, the observed inhibition of proximal tubule bicarbonate reabsorption may reflect primarily the activation of PKA and consequent inhibition of the Na^+-H^+ exchanger. Recent studies, however, suggest the possibility of a more complex sequence of events. Hruska and associates reported that parathyroid hormone induces a transient increase in the cytosolic concentration of calcium in renal proximal tubule cells [51]. The same laboratory also reported that parathyroid hormone activates phospholipase C and the phosphoinositide signal transduction pathway in these cells [52]. The ability of parathyroid hormone to alter the cell concentration of calcium and to activate phospholipase C raises the interesting possibility that the calcium-dependent protein kinases may modulate or act in concert with PKA to regulate the activity of the Na^+-H^+ exchanger. Recent studies by Cole et al in opossum proximal convoluted tubule cells suggest that activation of either PKA or PKC inhibits the sodium dependent uptake of phosphate [53]. At physiologic concentrations of parathyroid hormone, the authors suggest that activation of PKC, and not PKA via cAMP generation is the major mediator of the decrease in phosphate transport. One advantage of studies with the solubilized protein preparation is that each kinase can be examined independently. Integration of such findings into a model for understanding the function of intact cells, however, must be done with caution. For example, the concentrations of the protein kinases employed in the in vitro studies with solubilized proteins may not be physiologic. In addition, we have not yet examined the interactions between the kinases in this system, that is, the effect of protein phosphorylation by two or more kinases acting simultaneously or sequentially. Finally, as noted earlier, the complex interaction between the protein kinases themselves in intact cells remains ill defined.

Summary and conclusions

Starting from observations in intact cells and extending to studies in native membranes and solubilized membrane proteins, a significant body of evidence has been accumulated to indicate that some of the short-term regulatory influences on the Na^+-H^+ exchanger in the apical membrane of the proximal convoluted tubule act via protein phosphorylation mediated by specific protein kinases. Protein phosphorylation mediated by PKA inhibits the Na^+-H^+ exchanger while that mediated by PKC stimulates activity. The effect of PKA and PKC on the Na^+-H^+ exchanger in native membranes and in solubilized brush border membrane proteins appears to be consistent with most of the published observations in intact cells. Further studies using solubilized, renal brush border membrane proteins indicated that protein phosphorylation mediated by CaM-kinase II inhibited the activity of the Na^+-H^+ exchanger. The physiologic significance of this observation in intact cells remains to be determined. It is hoped that the types of experimental approaches outlined in this review will yield additional insights into the structure of the Na^+-H^+ exchanger and to a clearer understanding of its physiologic regulation.

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Acknowledgments

The authors acknowledge the technical assistance of Deborah Steplock and Quang Dinh and the secretarial skills of Ana Thannoun. This work was supported by grants from The National Institutes of Health (DK 37319 and DK 36466), The American Heart Association, Texas Affiliate, Travenol, Inc, and Squibb Pharmaceutical Inc.

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